Briefly, the present invention relates to methods of selecting an anti-aggregation molecule having the chaperone-like activity of anti-aggregation, methods of preventing or reducing aggregation, or disaggregating aggregates of an aggregating protein in subjects which do not yet have a protein aggregation disease, methods for treatment of a protein aggregation disease, and pharmaceutical compositions for prevention or treatment of a protein aggregation disease. The methods involve administering an anti-aggregation molecule, such as a monoclonal antibody that is capable of binding to a bioactive native aggregating protein or an aggregated form thereof, or administering an expression vector that encodes such an anti-aggregation molecule.

In compliance with 37 C.F.R. §1.173(c), the following statements are made. Patent claims 1-4 and added claims 5-9, 16-25 and 88-125 are pending. Originally added claims 10-15 and 26-87 have been cancelled. The following is an explanation of the support in the disclosure of the patent for the changes made to the claims.

In claim 5, the statement that the subject is one that does not have a protein aggregation disease is implicit from the original disclosure, for example, at column 6, lines 1-6, that the present invention is for use "to prevent or reduce protein aggregation in vivo". See also column 1, lines

5-9, and column 5, lines 39-41. Thus, it is an implicit disclosure that, when an invention is being used for prevention, it is administered to a subject which does not yet have the disease. The deletion of "being non-inhibitory to the biological activity of said aggregating protein", in claim 5 as well as in claims 16 and 21, does not create a written description problem and is supported, for example, in the paragraphs at column 3, lines 24-32, where it is indicated that the requirement for allowing the aggregating protein to function is only applicable to enzymes. It certainly is not applicable to β-amyloid, which has no known function. The other changes to claim 5 are merely clarifying.

The amendments to claims 9, 20, 25, 92 and 98 are merely to correct reference to "luteinizing hormone releasing hormone", which is disclosed at column 5, line 66 -- the comma was misplaced.

The amendments to claim 16 are all merely clarifying or have otherwise already been discussed with respect to claim 5.

The amendments to claim 21 are supported for the same reasons as discussed above with respect to claim 5.

New claim 100 is supported by claim 24 of the parent application as originally filed, in combination with the list of self-aggregating peptides or proteins at column 5, lines

61-67, of the present specification. The dependent claims are based on the individual proteins of the same paragraph of column 5.

Claim 107 is based on claim 7 of the parent application as originally filed, in combination with the same list of self-aggregating peptides or proteins at column 5, lines 61-67. Again, all of claims 108-125 are based on the same paragraph.

As to the amendments to the specification, the amendments to column 1, line 27; column 2, line 59; column 4, lines 49 and 50; column 6, line 62; column 7, lines 13, 31, 37, 45, 54 and 63; column 8, lines 5 and 27; and column 13, lines 22, 26 and 42, are all corrections of errors made by the Patent and Trademark Office in the printing of the patent. The correct version is present and therefore supported in the parent application as filed.

As to the remaining changes, the changes at column 2, lines 65 and 66, are merely grammatical changes to correct subject and verb correspondence. The correction to column 3, line 44, is merely another grammatical correction. The correction to column 4, line 6, corrects an obvious spelling error. The correction to column 4, lines 18 and 19, merely make the description of Figure 4 correspond to Figure 4. The nature of the hatching was changed when formal drawings were

filed in the parent case, and the description of the bars was inadvertently not changed when the formal drawings were filed, at least insofar as Figure 4 is concerned. The correctness of the changes is self-evident. With respect to column 4, line 33, there is only one bar with a left-slanting diagonal line and showing an amount of 60%. Thus, this correction is merely grammatical. At column 6, line 65, the correction is of an obvious typographical error made in the original application. The correction to column 11, line 17, is an obvious correction to make the buffer in this paragraph correspond to the description of the same buffer at column 11, line 8. While the printed patent had "Hl", this was a printing error on the part of the PTO as the original application had "Hcl". This, too, was incorrect as obviously "HCl", i.e., hydrochloric acid, was intended. The corrections at column 11, lines 23, 26 and 29, are all obvious corrections of typographical errors, as are the corrections at column 12, lines 29, 51 and 58, and column 13, line 21.

In the official action of June 29, 2001, the examiner stated that in applicant's response of January 8, 2001, the paragraph bridging pages 3 and 4 is unclear as the application as filed appeared to include an offer to surrender the patent and a consent of assignee, accompanied by a

certificate under 37 C.F.R. §3.73(b), which appeared to be proper. The examiner has requested clarification.

Applicant apologizes for the confusion which has been engendered by the papers as originally filed with this case. It is the understanding of the undersigned that on November 16, 1999, the following papers were filed in the Patent and Trademark Office.

(1) Form entitled "Reissue Patent Application Transmittal" (1 page);

(2) Form entitled "Reissue Application Fee Transmittal Form" (1 page);

(3) Form entitled "Reissue Application Declaration by the Inventor" (signed by the inventor in Hebrew) (3 pages, including one unnumbered paged referred to as an attachment to page 1);

(4) Form entitled "Reissue Application Declaration by the Assignee" (3 pages, including one unnumbered page referred to as an attachment to page 1);

(5) Form entitled "Reissue Application by the Inventor, Offer to Surrender Patent" (which includes as part of the form a consent of the assignee) and including a form entitled "Certificate under 37 C.F.R. §3.73(b)";

(6) Preliminary Amendment (including new claims 5-87);

(7) Specification and claims, including a cover page with only the top line of the printed patent, each of columns 1-18 of the printed patent and the abstract with a proposed amendment therein;

(8) Copies of the figures from the printed patent.

It is apparent that, in view of the declaration signed by the inventor that was originally filed, the "Reissue Application Declaration by the Assignee" should not have also been filed as only one such declaration is necessary. In view of the fact that the application was filed within two years of the issue date of the patent and that the claims added by preliminary amendment are broadening, patentee obviously intended that the "Reissue Declaration Signed by the Inventor" be the operative declaration. The sentence bridging pages 3 and 4 of applicant's amendment of January 8, 2001, requests that the declaration of the assignee be disregarded. The confusion noted by the examiner relates to the fact that applicant failed to note that the form relating to the offer to surrender the patent included a written consent of the assignee and a certification under §3.73(b). This is why a new consent of assignee was filed on May 25, 2001. The "Consent of Assignee" filed May 25, 2001, can be disregarded as redundant in view of the originally-filed consent of assignee, which the examiner concedes to be proper.

The previous confusion is regretted. It is hoped that this detailed explanation, together with the attached substitute declaration, discussed below, will clarify matters.

Claims 5-99 have been rejected under 35 U.S.C. §251 as being improperly broadened in a reissue application made and sworn to by the assignee and not the patentee, and claims 1-99 have been rejected as being based upon a defective reissue declaration under 35 U.S.C. §251. The examiner states that the declaration signed by the inventor is of poor quality, and the pagination is incorrect and confusing. Further, the examiner points out that the inventor's signature appears to be different in this and in the parent case. The examiner has required that applicant file a single, complete and unaltered declaration document in compliance with 37 C.F.R. §§1.63 and 1.175.

Attached hereto is a paper entitled "Substitute Reissue Declaration under 37 C.F.R. §1.175 and Power of Attorney for Reissue of Letters Patent 5,688,651. This four-page document is signed by Prof. Beka Solomon, both in English (as she signed the declaration in the parent case) and in her native Hebrew (as she signed the declaration as originally filed in this case). Please substitute this reissue declaration for all declarations previously filed in this case. Please disregard all previous copies of the explanation

of error. It is submitted that this declaration fully complies with 35 U.S.C. §251 and, as the declaration is signed by the inventor, the claims are not being improperly broadened. Reconsideration and withdrawal of these rejections and acceptance of the present application as having been properly filed, as all requirements of 35 C.F.R. §1.171-175 have been fully complied with, are respectfully urged.

Claims 5, 9-11, 15, 16, 20 (which are all in part drawn to antibody), 6-8, 12-14, 17-19, 21-52 and 85 (in part drawn to antibody) have been rejected under 35 U.S.C. §251 as being an improper recapture of claimed subject matter deliberately cancelled in the application for the patent upon which the present reissue is based. The examiner states that the instant claims 5-52 and 85 correspond in subject matter to Groups II and III of the parent application, which had been restricted out and cancelled in the parent application. More specifically, the examiner states that claims 21-52 are drawn to the same subject matter as the original claims of Group II of the parent case, and claims 5-20 and 85 are drawn to the same subject matter as the original claims of Group III of the parent case. The examiner states that the failure to file a divisional application is not considered to be an error that can be corrected by filing a reissue application. This rejection is respectfully traversed.

Claim 5, as presently amended, is not drawn to the same subject matter as original claim 24 in the parent case, which was made part of Group III by the examiner during the prosecution of the application which led to the patent presently in reissue. Group III included only claim 24 of the original application. This claim reads as follows:

> 24. The method of treating a protein aggregation disease including the steps of
>
> preparing at least one human monoclonal antibody that binds to an aggregated protein which is the cause of a disease and which reverses aggregation allowing bioactivity, and
>
> administering the monoclonal antibody.

It is, thus, clear that claim 24 is directed only to a method of treating a disease. Claim 5, on the other hand, is directed to a method of preventing a disease, and it specifically states that the anti-aggregation molecule is administered to "a subject that does not have a protein aggregation disease". A claim drawn to prevention is not directed to the same invention as a claim drawn to treatment of a disease. Accordingly, newly-amended claim 5 and those claims dependent therefrom are not subject to recapture estoppel. Claim 5 is not "of the same or broader scope than those claims that were cancelled from the original application" (*Ball Corporation v. United States*, 221 USPQ 289,

295 (Fed Cir 1984)). The claim does not even overlap with claim 24 as originally submitted. Accordingly, claim 5, as presently amended, should not be included within that group of claims which the examiner has stated is subject to recapture estoppel, but should be included among the claims listed in section 7 of the Office action, which are not directed to the same invention as any of the claims that were originally filed in the application which led to the patent which is the subject of the present reissue application.

Similarly, claim 21 is not drawn to the same invention as any of claims 7-23 of the original claims (designated Group II in the original restriction requirement) as the methods of all of claims 7-18 are directed to "treating a protein aggregation disease". Claim 21 is only directed to prevention of aggregation and reducing aggregation or disaggregating preaggregated aggregates in "a subject that does not have a protein aggregation disease". Accordingly, the invention of presently amended claim 21, and those claims dependent therefrom, should be considered to be independent and distinct from the invention of original claims 7-23.

The examiner states that claim 16 is drawn to the same subject matter as the original claim 24 of Group III. However, claim 16 is drawn to a pharmaceutical composition of an anti-aggregation molecule capable of preventing and

reducing aggregation of aggregating protein and disaggregating aggregates of aggregating protein, along with a pharmaceutically acceptable carrier. No claims to such a pharmaceutical composition were present in the 24 claims as originally submitted in the parent case. The only composition claims contained an expression vector, not the anti-aggregation molecule *per se*. Compositions containing the anti-aggregation molecule *per se* are supported, for example, at column 9, lines 24-28, of the present specification. As claims to this invention were never present in the original application (due to error without deceptive intent) and are not of the same or broader scope than those claims that were cancelled from the original application, they should not be subject to recapture estoppel. Accordingly, claim 16 and those claims dependent therefrom, including new claims 120-125, are properly examinable in the present reissue application.

Furthermore, claim 24 as originally submitted in the parent case was broadly directed to the genus of human monoclonal antibodies that bind to any aggregated protein which is the cause of a disease and which antibody reverses aggregation. New claim 100 has now been added which specifies a Markush group of particular aggregating proteins. Furthermore, dependent claims 101 to 106 are directed to

specific species of aggregating proteins, including β-amyloid, carboxypeptidase A, amylin, bombesin, etc. Thus, claim 100 is not of the same or broader scope than those claims that were cancelled from the original application. No species claims were present with respect to the method of claim 24 as originally presented in the parent case. If the examiner considers these claims to be drawn to multiple independent and distinct species, then whatever species is elected should be considered to be independent and distinct from claim 24 as originally submitted in the parent case. The same is true with respect to new claims 120-125, ultimately dependent from claim 16 herein. While the language of 37 C.F.R. §1.176 which is applicable to this case (quoted and discussed below) does not permit the examiner to require division, 37 C.F.R. §1.177 (in its present form[1] and in the form prior to the November 8, 2000, revision) specifically permits the voluntary filing of such a divisional by the reissue applicant. See §§1450-1451 of Rev. 1, 7th Edition, MPEP (Feb. 2000), attached hereto, for the practice under the old rule §1.176 under which the present application is being

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[1] Note that FR 65:54645 states:

> The changes to §1.177 relating to divisional reissues are effective on the date of publication of the rule in the **Federal Register** [September 8, 2000] for all pending and new reissue applications.

examined. If the examiner chooses to make a species election requirement in this case among the species enumerated in claim 100 and/or claim 20, applicant will voluntarily restrict the claims to the elected species and retain the option of filing a divisional on other species at an appropriate time.

The same logic applies for new claim 107, which is directed to a Markush group of aggregating proteins, and dependent claims 108-113, drawn to specific ones. These claims are different from any of the claims originally submitted in the parent case. Claim 7 as originally filed in the parent case was broadly drawn to any anti-aggregation molecule that binds to any aggregating protein. It should be noted that none of the claims as originally submitted in the parent case depended from claim 7 of the parent case. Claim 9 as originally filed in the parent case, which is specifically directed to anti-β-amyloid proteins, was dependent from claim 5 of the parent case, which is in Group I, and not from claim 7 of the parent case, which is in Group II of the restriction requirement made in the original application. Accordingly, if the examiner considers the species of the Markush group of claim 107 and of individual claims 108-113 to be drawn to multiple independent and distinct species, then whatever species is elected should be considered to be independent and distinct from claim 7, as originally submitted in the parent

case. Again, if the examiner chooses to make a species election requirement in this case among the species enumerated in claim 107, applicant will voluntarily restrict the claims to the elected species and retain the option of filing a divisional on other species at an appropriate time.

Claims 10-15 and 26-87 have now been deleted in order to facilitate further prosecution of this case. None of the remaining claims are directed to claims of the same or broader scope than those claims which were cancelled from the original application and, therefore, prosecution thereof is not barred by the recapture rule. Reconsideration and withdrawal of this rejection insofar as it might apply to any of the presently amended claims are, therefore, respectfully urged.

The examiner states that newly-submitted claims 5 (in part), 11, 15, 16, 20, 53-75, 76-79 (in part), 80-84, 85 (in part) and 86-99 are directed to inventions that are independent or distinct from the invention originally claimed. The examiner's reference to "in part" is understood as meaning to the extent that they do not read on the situation where the anti-aggregation molecule is a monoclonal antibody. The examiner states that the claims to a method of selecting an anti-aggregation molecule in an *in vitro* assay have been constructively elected by original presentation for

prosecution on the merits in the parent case and, accordingly, the remaining claims have been rejected under 35 U.S.C. §251 as being directed to inventions that are not directed to the same general invention in that the claims are now directed to new inventions, citing MPEP §§1412.01 and 1412.03. Further, the examiner states that the claims are directed to inventions that broaden the scope of the inventions claimed in the original patent by claiming different inventions and, therefore, these claims are improperly broadening the inventions. This rejection is respectfully traversed.

First of all, with respect to the examiner's comment about the claims broadening the inventions, applicant agrees that the present claims broaden the inventions originally issued in the patent whose reissue is being sought by the present application. However, as the present application was filed within two years of the issue of that patent and as a properly executed declaration by the inventor is of record in this case, such broadened claims are permissible in accordance with the first paragraph of 37 C.F.R. §1.173(a).

To the extent that this rejection is effectively a restriction requirement, inviting applicant to file divisional applications, the examiner's attention is drawn to the fact that current Rule 37 C.F.R. §1.176 is <u>not applicable to the present application</u>. The current version of 37 C.F.R. §1.176

became effective on November 7, 2000, and states at §1.176(b) that restriction between subject matter of the original patent and previously unclaimed subject matter may be required and that, if such restriction is required, the subject matter of the original claims will be held to be constructively elected. However, the rule promulgation for the change that resulted in the presently-worded §1.176 explicitly states, at Federal Register 65:54644 (September 8, 2000):

> Elimination of the prohibition against restriction in divisional application under §1.176 is effective for reissue applications filed on or after the date that is 60 days after the date of publication in the **Federal Register.**

Thus, the new wording of 37 C.F.R. §1.176, which eliminates the prohibition against requiring divisional applications, is only effective for reissue applications filed on or after November 7, 2000. As the present application was filed on November 16, 1999, the version of 37 C.F.R. §1.176 which was in existence on that date is applicable to the examination of the present application. Old §1.176, which is applicable to the examination of the present application, states:

> An original claim, if re-presented in the reissue application, is subject to reexamination, and the entire application will be examined in the same manner as original applications, subject to the rules relating thereto, excepting that division will not be required.

Thus, under the rule applicable to this case, restriction requirements are improper. See MPEP §1450 of the 7th Edition, Rev. 1, Feb. 2000, attached hereto, for a description of the practice under old rule §1.176, which is applicable in this case. As the examiner has apparently taken the position that the claims subject to the rejection in section 7 of the official action are not subject to the recapture estoppel rejection of section 6 of the official action, all of these claims must now be examined on the merits by the examiner. Accordingly, reconsideration and withdrawal of this rejection (restriction requirement) and examination on the merits of all of the claims now present in the case are respectfully urged.

The examiner has noted that text is missing from pages 2, 3, 5, 10, 13, 14, 16, 17 and 18 at the top due to improper margin size. The examiner has requested that the missing text be submitted in the form of substitute pages or a substitute specification in its entirety with proper margins.

Attached hereto is a substitute specification, complying with present Rule 37 C.F.R. §1.173(a)(1) in double-column format with the proper margins, including the amended abstract. Please substitute this specification for that originally submitted with the case. Applicant certifies that, except for the differences in the margins and the format layout, the specification submitted herewith is identical to

the specification as originally filed in the case and, therefore, no new matter is present.

It is noted that the examiner has indicated that claims 1-4 would be considered allowable if the new substitute Declaration is submitted. As the new substitute Declaration has been submitted, official acknowledgement of the allowability of claims 1-4 is respectfully urged.

It is submitted that all of the claims now present in the case fully comply with 35 U.S.C. §251. As restriction requirements are impermissible in reissue applications filed prior to November 7, 2000, such as the present one, and as none of the present claims are subject to recapture estoppel for the reasons discussed above, prompt consideration on the merits and allowance of all the claims now present in the case are earnestly solicited.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Atty. Docket: SOLOMON=1R
Beka SOLOMON	)	
U.S. Patent No.: 5,688,651	)	Washington, D.C.
Patented: November 18, 1997	)	
(U.S. Appln. No. 08/358,786 filed December 16, 1994)	)	August 1, 2001
For: PREVENTION OF PROTEIN AGGREGATION	)	

SUBSTITUTE REISSUE DECLARATION UNDER 37 C.F.R. §1.175 AND POWER OF ATTORNEY FOR REISSUE OF LETTERS PATENT 5,688,651

Honorable Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Beka Solomon, hereby solemnly declare that:

I am a citizen of Israel and my residence and post office address are as stated below next to my name.

I verily believe myself to be the original, first and sole inventor of the subject matter which is described and claimed in United States Letters patent 5,688,651, granted on November 18, 1997, and for which a reissue patent is sought on the invention entitled PREVENTION OF PROTEIN AGGREGATION, the specification of which was filed on November 16, 1999, as reissue application no. 09/441,140 and was amended on November 16, 1999, and on January 8, 2001.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by the amendments referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56.

I verily believe the original Letters Patent 5,688,651, to be wholly or partly inoperative or invalid by reason of the patentee claiming more or less than it had the right to claim in the patent.

At least one error upon which reissue is based is the following:

At the time the patent was in prosecution, all parties involved, i.e., Applicant, Assignee and the attorney involved in the prosecution of the patent application, were concentrating their efforts on the patenting of the presently claimed method of selecting an anti-aggregation molecule having the chaperone-like activity of anti-aggregation that issued in the 5,688,651 patent as claims 1-4, the only claims in the patent.

It was only recently that the Applicant/Patentee and the Assignee realized that the concepts fully disclosed in the specification directed to pharmaceutical compositions for preventing or reducing aggregation of an aggregating protein or for disaggregating preaggregated aggregates of the

aggregating protein and methods of disaggregating an aggregate of or preventing the aggregation of an aggregating protein by causing an anti-aggregation molecule to come into contact with the aggregate or with the soluble aggregating protein were not claimed *per se*, and further they could be and should be now so claimed.

All errors corrected in this reissue application arose without any deceptive intention on the part of the applicant.

As the named inventor, I hereby appoint the following registered practitioners to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith:

All of the practitioners associated with Customer Number 001444.

Direct all correspondence to the address associated with Customer Number 001444, which is presently:

BROWDY AND NEIMARK, P.L.L.C.
624 Ninth Street, N.W.
Washington, D.C. 20001-5303
(202) 628-5197.

The undersigned hereby authorizes the U.S. attorneys or agents appointed herein to accept and follow instructions from RAMOT UNIVERSITY AUTHORITY FOR APPLIED RESEARCH AND INDUSTRIAL DEVELOPMENT LTD. as to any action to be taken in the U.S. Patent and Trademark Office regarding this

application without direct communication between the U.S. Attorneys or Agents and the undersigned. In the event of a change of the persons from whom instructions may be taken, the U.S. Attorneys or Agents appointed herein will be so notified by the undersigned.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 21/8/2001 By: Beka Solomon

Beka SOLOMON

Residence: Herzlya Pituach, ISRAEL
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application solely to provoke an interference proceeding before the PTO because it did not assert that there was any error as required by 35 U.S.C. 251 in the patent.). A reissue application can be employed to provoke an interference if the reissue application:

(A) adds copied claims which are not present in the original patent;

(B) amends claims to correspond to those of the patent or application with which an interference is sought; or

(C) contains at least one error (not directed to provoking an interference) appropriate for the reissue.

In the first two situations, the reissue oath/declaration must assert that applicant erred in failing to include claims of the proper scope to provoke an interference in the original patent application. Note that in *In re Metz*, 1998 U.S. App. LEXIS 23733 (Fed. Cir. 1998)(unpublished), the Federal Circuit permitted a patentee to file a reissue application to copy claims from a patent in order to provoke an interference with that patent. Furthermore, the subject matter of the copied or amended claims in the reissue application must be supported by the disclosure of the original patent under 35 U.S.C. 112, first paragraph. See *In re Molins*, 368 F.2d 258, 261, 151 USPQ 570, 572 (CCPA 1966) and *In re Spencer*, 273 F.2d 181, 124 USPQ 175 (CCPA 1959).

A reissue applicant cannot present added or amended claims to provoke an interference if the claims were deliberately omitted from the patent. If there is evidence that the claims were not inadvertently omitted from the original patent, e.g., the subject matter was described in the original patent as being undesirable, the reissue application may lack proper basis for the reissue. See *In re Bostwick*, 102 F.2d at 889, 41 USPQ at 282 (CCPA 1939)(reissue lacked a proper basis because the original patent pointed out the disadvantages of the embodiment that provided support for the copied claims).

The issue date of the patent with which an interference is sought must be less than 1 year prior to the presentation of the copied or amended claims in the reissue application. See 35 U.S.C. 135(b) and MPEP § 715.05 and § 2307. If the reissue application includes broadened claims, the reissue application must be filed within two years from the issue date of the original patent. See 35 U.S.C. 251 and MPEP § 1412.03.

REISSUE APPLICATION FILED WHILE PATENT IS IN INTERFERENCE

If a reissue application is filed while the original patent is in an interference proceeding, the reissue applicant is required to notify the Board of Patent Appeals and Interferences of the filing of the reissue application within 10 days from the filing date. See 37 CFR 1.660(b) and MPEP § 2360.<

1450 Restriction and Election of Species

The examiner may **not** require restriction in a reissue application (37 CFR 1.176 and MPEP § 1440). Even where the original patent contains claims to different inventions which the examiner considers independent and distinct, and the reissue application claims the same inventions, the examiner should not require restriction between them or take any other action with respect to the question of plural inventions. Restriction may only be requested by the applicant (37 CFR 1.177 and MPEP § 1451). In situations where a reissue applicant presents claims for the first time that are distinct and separate from the claims of the patent, the examiner must follow the practice resulting from *In re Amos*, 953 F.2d 613, 618, 21 USPQ2d 1271, 1274 (Fed. Cir. 1991) as set forth in MPEP § 1412.01.

A reissue applicant's failure to timely file a divisional application is not considered to be error causing a patent granted on elected claims to be partially inoperative by reason of claiming less than the applicant had a right to claim. Thus, such error is not correctable by reissue of the original patent under 35 U.S.C. 251. *In re Watkinson*, 900 F.2d 230, 14 USPQ2d 1407 (Fed. Cir. 1990); *In re Orita*, 550 F.2d 1277, 1280, 193 USPQ 145, 148 (CCPA 1977). See also *In re Mead*, 581 F. 2d 251, 198 USPQ 412 (CCPA 1978). Likewise, if the original patent specification shows an intent not to claim the newly presented invention, that invention cannot be added by reissue. In these situations, the reissue claims should be rejected under 35 U.S.C. 251 for lack of defect in the original patent and lack of error in obtaining the original patent. See also MPEP § 1412.01.

When the original patent contains claims to a plurality of species and the reissue application contains claims to the same species, election of species should not be required even though there is no allowable generic claim. If the reissue application presents claims to species not claimed in the original patent, election of species should not be required, but the added claims may be rejected, where appropriate, for lack of defect in the original patent and lack of error in obtaining the original patent as discussed above.

1451 Divisional Reissue Applications; Continuation Reissue Applications Where the Parent is Pending [R-1]

35 U.S.C. 251. Reissue of defective patents.

*****

The Commissioner may issue several reissued patents for distinct and separate parts of the thing patented, upon demand of the applicant, and

upon payment of the required fee for a reissue for each of such reissued patents.

*****

37 CFR 1.177. Reissue in divisions.

The Commissioner may, in his or her discretion, cause several patents to be issued for distinct and separate parts of the thing patented, upon demand of the applicant, and upon payment of the required fee for each division. Each division of a reissue constitutes the subject of a separate specification descriptive of the part or parts of the invention claimed in such division; and the drawing may represent only such part or parts, subject to the provisions of §§ 1.83 and 1.84. On filing divisional reissue applications, they shall be referred to the Commissioner. Unless otherwise ordered by the Commissioner upon petition and payment of the fee set forth in § 1.17(i), all the divisions of a reissue will issue simultaneously; if there is any controversy as to one division, the others will be withheld from issue until the controversy is ended, unless the Commissioner orders otherwise.

Questions relating to the propriety of divisional reissue applications and continuation reissue applications should be referred via the Group Special Program Examiner to the Special Program Law Office.

DIVISIONAL REISSUE APPLICATIONS

As is pointed out in MPEP § 1450, the examiner cannot require restriction in a reissue application; only applicant can initiate a division of the claims by demand in accordance with 37 CFR 1.177. Where the original patent claims contain several independent and distinct inventions, they can be divided into separate divisional reissues if the applicant demands it. 37 CFR 1.177 sets forth a possibility for filing divisional reissue applications.

When divisional reissue applications are filed, appropriate amendments to the continuing data entries are to be made to the first sentence of the specification, and to the file wrappers, for all such applications, so that **>adequate notice is provided that more than one reissue application has been filed for a single original patent.

The provisions of 37 CFR 1.177 currently require that all divisional reissue applications issue simultaneously. However, in view of *In re Graff*, 111 F.3d 874, 42 USPQ2d 1471 (Fed. Cir. 1997), Office policy has been modified. The requirement of 37 CFR 1.177 for simultaneous issuance will be routinely waived *sua sponte* (under 37 CFR 1.183) prior to examination of the reissue applications in a letter issued by the Special Program Law Office under 37 CFR 1.177. Accordingly, where an examiner becomes aware of the existence of divisional reissue applications, the examiner should check to ensure that a 37 CFR 1.177 letter issued by the Special Program Law Office is present in each divisional reissue file. If such a letter is not present, the divisional reissue applications should be immediately forwarded via the Group Special Program Examiner to the Special Program Law Office.<

Situations yielding divisional reissues occur infrequently and usually involve only two such files. It should be noted, however, that in rare instances in the past, there have been more than two (and as many as five) divisional reissues of a patent.

CONTINUATION REISSUE APPLICATIONS

A continuation of a reissue is ** not >ordinarily filed< "for distinct and separate parts of the thing patented" as called for in the second paragraph of 35 U.S.C. 251. The decision of *In re Graff*, 111 F.3d 874, 42 USPQ2d 1471 (Fed. Cir. 1997) interprets 35 U.S.C. 251 to permit multiple reissue patents to issue even where the multiple reissue patents are not for "distinct and separate parts of the thing patented." The court stated:

> Section 251[2] is plainly intended as enabling, not as limiting. Section 251[2] has the effect of assuring that a different burden is not placed on divisional or continuation reissue applications, compared with divisions and continuations of original applications, by codifying the Supreme Court decision which recognized that more than one patent can result from a reissue proceeding. Thus § 251[2] places no greater burden on Mr. Graff's continuation reissue application than upon a continuation of an original application; § 251[2] neither overrides, enlarges, nor limits the statement in § 251[3] that the provisions of Title 5 apply to reissues.

111 F.3d at 877, 42 USPQ2d at 1473. Accordingly, >prosecution of< a continuation of a reissue application will be permitted ** (despite the presence of the parent reissue) where the continuation complies with the rules for reissue.

The parent and the continuation reissue applications should be examined together if possible. An appropriate amendment to the continuing data entries is to be made to the first sentence of the specification, and to the file wrappers, for ***both the parent and the continuation*** reissue applications, so that the parent-continuation relationship of the reissue applications is specifically identified and notice is provided of both reissue applications.

Where the parent reissue application issues prior to the examination of the continuation, the claims of the continuation should be carefully reviewed for double patenting over the claims of the parent. Where the parent and the continuation reissue applications are examined together, a provisional double patenting rejection should be made in both cases as to any overlapping claims. See MPEP § 804 - § 804.04 as to double patenting rejections.>Any terminal disclaimer filed to obviate an obviousness-type double patenting rejection ensures common ownership of the reissue patents throughout the remainder of the unexpired term of the original patent.<

If the parent reissue application issues without any cross reference to the continuation, amendment of the parent reissue patent to include a cross-reference to the

continuation should be required **>by Certificate of Correction<.

1453 Amendments to Reissue Applications

37 CFR 1.121. Manner of making amendments.

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(b) *Amendments in reissue applications*: Amendments in reissue applications are made by filing a paper, in compliance with § 1.52, directing that specified amendments be made.

(1) *Specification other than the claims*: Amendments to the specification, other than to the claims, may only be made as follows:

(i) Amendments must be made by submission of the entire text of a newly added or rewritten paragraph(s) with markings pursuant to paragraph (b)(1)(iii) of this section, except that an entire paragraph may be deleted by a statement deleting the paragraph without presentation of the text of the paragraph.

~~(ii) The precise point in the specification must be indicated~~ where the paragraph to be amended is located.

(iii) Underlining below the subject matter added to the patent and brackets around the subject matter deleted from the patent are to be used to mark the amendments being made.

(2) *Claims*. Amendments to the claims may only be made as follows:

(i)(A)The amendment must be made relative to the patent claims in accordance with paragraph (b)(6) of this section and must include the entire text of each claim which is being amended by the current amendment and of each claim being added by the current amendment with markings pursuant to paragraph (b)(2)(i)(C) of this section, except that a patent claim or added claim should be cancelled by a statement cancelling the patent claim or added claim without presentation of the text of the patent claim or added claim.

(B) Patent claims must not be renumbered and the numbering of any claims added to the patent must follow the number of the highest numbered patent claim.

(C) Underlining below the subject matter added to the patent and brackets around the subject matter deleted from the patent are to be used to mark the amendments being made. If a claim is amended pursuant to paragraph (b)(2)(i)(A) of this section, a parenthetical expression "amended," "twice amended," *etc.*, should follow the original claim number.

(ii) Each amendment submission must set forth the status (*i.e.*, pending or cancelled) as of the date of the amendment, of all patent claims and of all added claims.

(iii) Each amendment when originally submitted must be accompanied by an explanation of the support in the disclosure of the patent for the amendment along with any additional comments on page(s) separate from the page(s) containing the amendment.

(3) *Drawings*.

(i) Amendments to the original patent drawings are not permitted. Any change to the patent drawings must be by way of a new sheet of drawings with the amended figures identified as "amended" and with added figures identified as "new" for each sheet changed submitted in compliance with § 1.84.

(ii) Where a change to the drawings is desired, a sketch in permanent ink showing proposed changes in red, to become part of the record, must be filed for approval by the examiner and should be in a separate paper.

(4) The disclosure must be amended, when required by the Office, to correct inaccuracies of description and definition, and to secure substantial correspondence between the claims, the remainder of the specification, and the drawings.

(5) No reissue patent shall be granted enlarging the scope of the claims of the original patent unless applied for within two years from the grant of the original patent, pursuant to 35 U.S.C. 251. No amendment to the patent may introduce new matter or be made in an expired patent.

(6) All amendments must be made relative to the patent specification, including the claims, and drawings, which is in effect as of the date of filing of the reissue application.

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The provisions of 37 CFR 1.121(b) apply to amendments in reissue applications. The practice outlined in this section must be complied with for any amendment submitted in a reissue application on or after December 1, 1997.

Amendments submitted before December 1, 1997 (under the prior practice) need not, and should not, be re-submitted under the current practice. However, if an amendment *is in fact re-submitted*, ~~it will be entered, unless non-entry is~~ directed or approved by the SPE or SPRE.

THE SPECIFICATION

37 CFR 1.121(b)(1) relates to the manner of making amendments to the specification other than the claims. It is not to be used for making amendments to the claims or the drawings.

37 CFR 1.121 (b)(1)(i) requires that all amendments which include any deletions or additions must be made by submission of a copy of each rewritten paragraph with markings (brackets and underlining), with the exception that an entire paragraph of specification text may be deleted by a statement deleting the paragraph *without presentation of the text of the paragraph.* 37 CFR 1.121 (b)(1)(i) also requires that all paragraphs which are *added* to the specification be submitted as completely underlined. In 37 CFR 1.121(b)(1)(ii), it is required that the precise point where each amendment is made must be indicated by applicant. 37 CFR 1.121(b)(1)(iii) defines the markings set forth in (b)(1)(ii) as being brackets for deletion and underlining for addition.

All bracketing and underlining is made in comparison to the original patent, not in comparison to the prior amendment.

Where a change is made in one sentence, paragraph or page, and the change increases or decreases the size of the sentence, paragraph or page, this will have no effect on the body of the reissue specification. This is because all insertions are made as blocked additions of paragraphs, which are not physically inserted within the specification papers. Rather, each blocked paragraph is assigned a letter and number, and a caret written in the specification papers indicates where the blocked paragraph is to be incorporated. In view of this, a reissue applicant need not be concerned with page formatting considerations when presenting amendments to the Office.

PREVENTION OF PROTEIN AGGREGATION

TECHNICAL FIELD

The present invention relates to the use of monoclonal antibodies, genetically engineered antibody fragments and small peptides which mimic antigen binding sites on the antibody for the prevention of protein aggregation in vivo and in vitro.

BACKGROUND OF THE INVENTION

When proteins are synthesized they generally must fold and assemble into a three dimensional form to be active. Initially, it was thought that proper folding was inherent in the amino acid sequence. Recent work has shown that additional proteins, now referred to as molecular chaperones, are required to mediate the folding process or unregulated aggregation of the polypeptides will occur preventing the formation of functional proteins (Goloubinoff et al., 1989; Welch, 1993). However, despite the existence of chaperones, aggregation of protein still occurs in vivo and can contribute to, or cause, various disease states.

Other factors must contribute to the occurrence of aggregation. These factors can include mutations of naturally occurring chaperones inhibiting function or allowing function with low efficiency (Wetzel, 1994). Further, "pathological", chaperones have been found which have been defined as "a group of unrelated proteins that induce beta-pleated conformation in amyloidogenic polypeptides" (Wisniewski and Frangione, 1992). It would be useful to be able to replace or augment the activity of the chaperones where necessary and to counteract the activity of pathological chaperones when present.

Protein aggregation is of major importance in biotechnology for the in vitro production of recombinant proteins. In vitro aggregation limits the protein stability, solubility and yields in production of recombinant proteins. In cells during production of recombinant proteins, aggregation is a major impediment of recombinant proteins leading to formation of inclusion bodies in the host cells (DeYoung et al, 1993; Wetzel, 1994; Vandenbroeck et al., 1993).

Further, in vivo protein aggregation or precipitation is the cause, or an associated pathological symptom, in amyloid diseases such as Down's syndrome, Alzheimer's disease, diabetes and/or cataracts, and in other disorders (DeYoung et al., 1993; Haass and Selkoe, 1993; Wetzel, 1994).

Several peptides including β-amyloid, have been shown to spontaneously self-associate, or aggregate, into linear, unbranched fibrils in serum or in isotonic saline (Banks and Kastin, 1992; Haass and Selkoe, 1993). At least fifteen different polypeptides are known to be capable of causing in vivo different forms of amyloidosis via their deposition in particular organs or tissues as insoluble protein fibrils. Iron, zinc, chromium or aluminum can participate in this aggregation (Bush et al., 1994).

Molecular chaperones were initially recognized as stress proteins produced in cells requiring repair. In particular, studies of heat shock on enzymes led the way to the discovery of molecular chaperones that function not only during cellular stress but normally to produce properly folded proteins. The heat shock model is still one of the models of choice in studying molecular chaperones (Welch, 1993; Goloubinoff et al., 1989).

Molecular chaperones are a ubiquitous family of proteins that mediate the post-translational folding and assembly of other unrelated proteins into oligomeric structures. They are

further defined as molecules whose functions are to prevent the formation of incorrect structures and to disrupt any that form. The chaperones non-covalently bind to the interactive surface of the protein. This binding is reversed under circumstances that favor the formation of the correct structure by folding. Chaperones have not been shown to be specific for only one protein but rather act on families of proteins which have the same stoichiometric requirements, i.e specific domains are recognized by chaperones. This does not provide the specificity required for therapeutic activity.

Further uses and descriptions of molecular chaperones are set forth in PCT published international patent application 93/11248, 93/13200, 94/08012 and 94/11513 incorporated herein by reference and in particular 94/08012 page 2 line 20 through page 5, line 14.

PCT published international patent application 93/11248 discloses the use of a chaperone in cell culture to promote efficient production of protein in transformed cells by co-expression of the chaperone molecule. This disclosure does not provide specificity as to which proteins are protected except through co-expression with the wanted protein nor does it provide information on how to use chaperones therapeutically.

PCT published international patent application 93/13200 discloses the use of a chaperone in a purification step for a recombinant protein isolated from a cell culture and also a fusion protein of the chaperone and recombinant protein. This disclosure also does not provide specificity as to which proteins are protected except through co-expression with the wanted protein nor does it provide information on how to use chaperones therapeutically.

PCT published international patent application 94/08012 discloses the use of a chaperone in cell culture to promote increased secretion of an overexpressed gene product in a host cell. This disclosure does not provide specificity as to which proteins are protected except through co-expression with the wanted protein nor does it provide information on how to use chaperones therapeutically.

PCT published international patent application 94/11513 discloses the use of a vector containing a molecular chaperone for treating neoplasms. This disclosure does not provide specificity as to which proteins are protected except through co-expression with the wanted protein nor does it provide information on how to use chaperones therapeutically to treat diseases or syndromes which involve protein aggregation.

In each of the aforementioned publications, the chaperones did not bind to native proteins and did not redissolve aggregated proteins.

Recent reports suggest that monoclonal antibodies (mAb) can have chaperone-like activity. The feasibility of using monoclonal antibodies to assist in the in vitro refolding process of guanidine-denatured S-protein was reported recently (Carlson and Yarmush, 1992). Previously, Blond and Goldberg (1987) used monoclonal antibodies as a tool in the identification and characterization of folding steps that involve the appearance of local native-like structures in B_2 subunit of tryptophansynthase. Since the mAb is epitope specific, the use of mAb provides more specificity than molecular chaperones. mAbs can be sought and engineered (Haber, 1992) that bind to the particular epitope in the protein of interest that is involved in the folding process.

The main difference between mAbs and molecular chaperones is that the latter does not bind to native proteins and is capable of interacting with many different polypeptide chains without exhibiting an apparent sequence preference

(Goloubinof et al., 1989). Moreover, chaperones suppress aggregation but do not redissolve aggregate already present. Similar behavior was recently reported for α-crystalin which, similar to other chaperones, does not react with active proteins, but forms a stable complex with denaturing or partially unfolded proteins, stabilizing against further aggregation (Rao et al., 1994).

Aggregated amyloid β-protein (βA4) is a major constituent of the abnormal extracellular amyloid plaque that characterizes the brains of victims of Alzheimer's disease (AD) (Haass and Selkoe, 1993). In vitro studies have shown that some of the metal ions found in biological systems, i.e. Fe, Al and Zn, can accelerate the aggregation process dramatically. The presence of "pathological" chaperones (Wisniewski and Frangione, 1992) and the above listed metals (Mantyh et al., 1993; Fraser et al., 1993) as proposed risk factors in Alzheimer's disease, favor β-amyloid cascade aggregation. If the interaction between the metal ion and the β-amyloid can be interrupted or prevented, then metal-induced aggregation can be reduced or eliminated. However, just binding a mAb at this site might prevent the metal-induced aggregation but would not allow normal functioning of the protein.

It would therefore be useful to develop the appropriate mAb with chaperone-like characteristics directed to the appropriate epitope on the β-amyloid molecule in order to prevent the accelerated metal-induced aggregation.

Further, it would be particularly useful to be able to develop a mAb as needed that prevents the aggregation of enzymes in vivo but that still allows the enzymes to function.

Still further, it is not always possible to isolate the appropriate chaperone for preventing aggregation of a molecule and to utilize it as a therapeutic. The availability of engineering and selecting mAbs and delivery systems for mAb makes it useful to develop specific mAb to serve as therapeutic chaperones.

SUMMARY OF THE INVENTION AND ADVANTAGES

According to the present invention, a method is provided of selecting anti-aggregation molecule such as a monoclonal antibody, a genetically engineered antibody fragment or a peptide which mimics the binding site of an antibody. These anti-aggregation molecules are able to bind to a native target molecule epitope with a high binding constant and must be non-inhibitory to biological activity of the target molecule.

The present invention further provides a method of treating a protein aggregation disease by creating an expression vector comprising nucleic acid including a sequence which encodes in expressible form the human form of the anti-aggregation molecule that binds to a native target molecule, an aggregating protein, and which prevents aggregation and allows biological activity of the target molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIG. 1 is a bar graph of the temperature-dependence of enzymic activity of Carboxypeptidase A; the residual enzymic activity of CPA after one hour incubation at increasing temperatures was measured using esterase substrate;

FIG. 2 is a bar graph of the time course of denaturation of Carboxypeptidase A after exposure at 50° C.; the residual esterase (single cross-hatch bars) and peptidase (open bars) enzymic activity of CPA was measured at two intervals of incubation at 50° C.; the amount of residual soluble enzyme was determined by sandwich ELIAS (bars of diagonal lines);

FIG. 3 is a bar graph of the enzymic activity of Carboxypeptidase A retained after exposure to 50° C. for one hour in the presence of monoclonal antibody CP_{10}; the immunocomplexation of CPA with increasing amounts of CP_{10} was performed before exposure at 50° C. for one hour; the residual peptidase (open bars) and esterase (single cross-hatch bars) enzymic activity of CPA was measured;

FIG. 4 is a bar graph of the effect of epitope location on the maintenance of the enzymic activity of heat-exposed Carboxypeptidase A; increasing amounts of monoclonal antibodies CP_{10} (single cross-hatch bars) and CP_9 (diagonal lines) and unrelated IgG (bars with diagonal lines) were added to CPA before exposure to 50° C. for one hour and esterase enzymic activity was measured;

FIG. 5 is a bar graph of the prevention of aggregation of Carboxypeptidase A by monoclonal antibody CP_{10}; aggregation of CPA, in the presence (bars with right slanting diagonal lines) and in the absence (single cross-hatch bars) of antibodies, was followed by determination of amount of mAb bound to coated CPA in a competitive ELISA; the absorbance at 495 nm obtained in the absence of added soluble CPA was set at 100% for bound antibody; the soluble CPA, before heat exposure, competes with the coated CPA for antibody binding, leading to decrease in amount of antibody bound (60%) (bars with left slanting diagonal lines);

FIG. 6 is a bar graph showing thermal aggregation of Carboxypeptidase A and its suppression by monoclonal antibodies CP_{10} and CP_9; aggregation of Carboxypeptidase A after exposure at 50° C. for one hour in the absence (open bars) of monoclonal antibodies and in the presence of CP_{10} (bars with diagonal lines) and CP_9 (double cross-hatch) was followed by determination of amount of antibody bound by sandwich ELISA; maximum binding (100%) was considered the amount of antibody bound to CPA before exposure to aggregation conditions;

FIGS. 7A and 7B are a pair of graphs (A and B) showing aggregation of β-amyloid (1–40) in the absence (diagonal lines bars) and in the presence (open bars) of monoclonal antibodies AMY-33 (A) and 6F/3D (B) followed by ELISA; (1) β-amyloid alone, (2) β-amyloid+50 MM heparan sulfate, (3) β-amyloid+10–3M $AlCl_3$; (4) β-amyloid+10^{-3}M $ZnCl_2$; and

FIG. 8 shows schematic diagram of β-amyloid (1–40) with horizontal lines representing the regions against which monoclonal antibodies were produced, vertical lines and shaded rectangular areas represent the heparan sulfate binding sites (residues 12–17, dark shaded), the proposed toxic fragment (residues 25–35) and the putative epitope of mAb AMY-33 (sequence 25–28, light shaded).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides a method of selecting monoclonal antibodies, genetically engineered antibody fragments and small peptides which mimic binding sites of the antibodies and which prevent aggregation and yet do not inhibit bioactivity. These anti-aggregation molecules with chaperone-like activity are able to bind to a native target

molecule epitope with a high binding constant and must be non-inhibitory to the biological activity of the target molecule. The method includes culturing an appropriate host cell transformed with DNA encoding the target molecule. The host cell chosen will express the target molecule in aggregated form. Examples of such cells are set forth in PCT published international patent application 93/11248, 93/13200 and 94/08012. Alternatively, the appropriate recombinant target molecule can be purchased or a naturally occurring molecule can be isolated or purchased.

The expressed target molecule is recovered and denatured. The denatured target molecule is mixed with the presumptive anti-aggregation molecule such as a monoclonal antibody, genetically engineered antibody fragment or small peptide which mimics an antibody binding site generally as set forth in PCT pending application 93/13200 and under conditions which allow for self-aggregation, temperature, pH or interaction with other aggregation-inducing agents. It is then determined if the mixture produces nonaggregated target molecules that are bioactive even in the presence of, and bound to, the presumptive anti-aggregation molecule.

In addition, the anti-aggregation molecule is screened for its ability to dissolve already aggregated proteins. The aggregated proteins are mixed with the anti-aggregation molecules under physiological conditions. It is then determined if the mixture produces nonaggregated target molecules that are bioactive even in the presence of, and bound to, the presumptive anti-aggregation molecule.

The antibodies, or peptide mimicking the binding site, must bind to an epitope on the target molecule which is a region responsible for folding or aggregation. In addition the anti-aggregation molecule is selected only if it does not show immune cross reactivity with other proteins with proximity to the target molecules under the same conditions employed in the bioactivity tests; that is, molecules which are found in the cell near the target molecule or molecules with sequences similar to the target molecules.

After the identification of the anti-aggregation molecules has been completed, it is possible to utilize two or more to prevent or reverse aggregation. They can be used concurrently to increase their chaperone-like effect, if their respective target epitopes are not overlapping and if, in binding to the target molecule, they do not interfere with each other.

Bioactivity is tested as is appropriate for the target molecule. For example, enzymatic activity of the target molecule for its substrate can be measured. Assays which measure in vitro enzymatic bioactivity are well known to those skilled in the art.

In the preferred embodiment of the method, the target molecule is β-amyloid and the monoclonal antibody is an anti-β-amyloid monoclonal. Alternatively, a genetically engineered antibody fragment as described hereinbelow can be used or a small peptide which mimics the antigen binding site of the antibody. The antigen binding site of an antibody can be determined from the DNA sequence of the respective CDR fragments.

The method has also been demonstrated with carboxypeptidase A as set forth in the Examples hereinbelow.

Other peptides or proteins with evidence of self aggregation can also be used in the present invention such as amylin (Young et al., 1994); bombesin, caerulein, cholecystokinin octapeptide, eledoisin, gastrin-related pentapeptide, gastrin tetrapeptide, somatostatin (reduced), substance P; and peptide, luteinizing hormone releasing hormone, somatostatin N-Tyr (Banks and Kastin, 1992).

Once an appropriate monoclonal antibody with chaperone-like activity is found or engineered or a peptide with the appropriate configuration, the present invention provides for its use therapeutically to prevent or reduce protein aggregation in vivo. In the preferred embodiment, the prevention of β-amyloid aggregation is undertaken.

A method of treating a protein aggregation disease intracellularly includes the steps of preparing (Haber, 1992; Harlow & Lane, 1988) or selecting an anti-aggregation molecule, such as a monoclonal antibody, genetically engineered monoclonal antibody fragment or peptide that mimics the binding site of an antibody, that binds to an aggregating protein which is the cause of a disease and which prevents aggregation and yet allows the protein to be bioactive. This molecule can be referred to as an anti-aggregation molecule with chaperone-like activity. An expression vector is created comprising nucleic acid including a sequence which encodes in expressible form the anti-aggregation molecule. The expression vector is then delivered to the patient.

In the preferred embodiment the human monoclonal antibody that binds to an aggregating protein and which prevents aggregation is utilized. In a further preferred embodiment the monoclonal antibody is an anti-β-amyloid and is designated AMY-33 which recognizes amino acids 1–28 of β-amyloid.

Work by Dueñas et al. (1994) and Marasco et al. (1993) have shown that single chain monoclonal antibodies are efficient for intracellular expression in eukaryotic cells. The single chain monoclonal antibody is composed of an immunoglobulin heavy chain leader sequence and heavy and light chain variable regions that are joined by an interchain linker. Marasco et al. (1993) have shown that such antibodies are not toxic to the cells and function when expressed in the cell.

The production of expression vectors is well known to those skilled in the art. In a preferred embodiment, the expression vector is constructed using the methodology as set forth by Dueñas et al. (1994), PCT pending application 94/11513. Methods not explicitly set forth are performed as generally set forth in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1992), and in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989).

Such vectors are known or can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors. Examples of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors can also contain elements for use in either procaryotic or eucaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

The expression vector can be a virus. Further the virus can be an RNA virus such as a disabled retro virus or a retrovital shuttle vector. The expression vector can also be vaccinia virus or an adenovirus. The expression vector can also be a plasmid. In a preferred embodiment wherein β-amyloid in the targeted molecule the expression vector is selected that is known to target the central nervous system.

In the present invention, the expression vector for use as a therapeutic agent comprises a nucleic acid including at least one sequence which encodes in expressible form an anti-aggregation molecule, which molecule binds to an aggregating protein that is the cause of a disease and which prevents aggregation but does not interfere with bioactivity. In a preferred embodiment the expression vector includes the sequence for a human monoclonal antibody that is an anti-β-amyloid monoclonal antibody with heparan-like characteristics. In a further preferred embodiment, the expression vector includes the sequence for the single chain monoclonal antibody of the above anti-β-amyloid mAb.

A specific example of DNA vital vector for introducing and expressing recombinant sequences is the adenovirus derived vector Adenop53TK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an adenovirus receptor. This vector as well as others that exhibit similar desired functions can be used to treat a mixed population of cells and can include, for example, an in vitro or ex vivo culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers that can be used to negatively select against cells infected with the recombinant virus such as antibiotic sensitivity. Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the vital vector or recombinant sequence, cellular transformation will not occur. Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

In addition, recombinant vital vectors are useful for in vivo expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original vital particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of vital vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. The vector to be used in the methods of the invention will depend on desired cell type to be targeted and will be known to those skilled in the art. For example, if breast cancer is to be treated then a vector specific for such epithelial cells would be used. Likewise, if diseases or pathological conditions of the hematopoietic system are to be treated, then a vital vector that is specific for blood cells and their precursors, preferably for the specific type of hematopoietic cell, would be used.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new vital particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed through subsequent rounds of infection. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

The expression vector containing the sequence for the anti-aggregation molecule may be administered to mammals, including humans, by any route appropriate to the condition being treated and in several ways. Suitable routes include oral, rectal, nasal, topical, vaginal and parenteral. It will be appreciated that the preferred route may vary with, for example, the condition of the recipient and the type of treatment envisaged.

If vital vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration can provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into a spinal fluid can also be used as a mode of administration, especially in the case of neuro-degenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection. Alternatively, the method as set forth by Tuomanen et al. (1993) can be used.

The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found described in Sambrook et al. and Ausubel et al., and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. Introduction of nucleic acids by infection offers several advantages over the other listed methods. Higher efficiency can be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types in vivo or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

An alternate mode of administration of the vector can be by direct inoculation locally at the site of the disease or pathological condition or by inoculation into the vascular system supplying the site with nutrients. Local administration is advantageous because there is no dilution effect and, therefore, a smaller dose is required to achieve expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects all cells in the inoculated area. If expression is desired in only a specific subset of cells within the inoculated area, then promoter and regulatory elements that are specific for the desired subset can be used to accomplish this

goal. Such non-targeting vectors can be, for example, viral vectors, viral genome, plasmids, phagemids and the like. Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

The expression vector of the present invention may be administered to the patient alone or in combination with liposomes or other delivery molecules. The expression vector is administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, and other factors known to medical practitioners. The "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve in the treated patients a reduction in protein aggregation and may also include but is not limited to improved survival rate, more rapid recovery, or improvement or elimination of symptoms and are selected as appropriate measures by those skilled in the art.

While it is possible for the expression vector to be administered alone, it is preferable to present it as a pharmaceutical formulation. The formulations of the present invention comprise at least one active ingredient: the monoclonal antibody or expression vector together with one or more pharmaceutically acceptable carriers and optionally other therapeutic ingredients. The carrier(s) must be acceptable in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipients. The carriers must also be selected so as not to interfere with the activity of the active ingredient.

The availability of monoclonal antibodies which bind to a specific antigen at distinct and well defined sites has led to a better understanding of the effects of highly specific enzyme-antibody interactions on the enzyme behavior. By appropriate selection it has been possible to isolate those antibodies that are non-inhibitory to biological activity of the enzyme and bind at "strategic locations" on the antigen molecule, resulting in a considerable stabilization effect of the enzyme conformation. Moreover, such monoclonal antibodies, when properly selected, prove to have a chaperone-like activity leading to a considerable refolding effect on the enzyme which was already partially heat denatured. In addition, the use of engineered monoclonal antibodies and their fragments, as well as peptides which mimic the binding site for the antigen on the antibody can be used in the present invention.

Carboxypeptidase A shows a decrease in solubility with an increase in temperature, accompanied by loss of enzymic activity and conformational changes leading to its aggregation. In the present study, the suppression of enzyme aggregation via its interaction with two monoclonal antibodies raised against native protein was investigated. ELISA measurements and determination of residual enzymic activity, as a probe of the native structure, were used to monitor the protein aggregation process. The studied monoclonal antibodies are non-inhibitory to the biological activity of the antigen or target molecule, bind on the strategic position on the molecule and proved to have a chaperone-like activity in the prevention of protein aggregation. The antibodies effect on the inhibition of aggregation was found to be related to the location of the antigenic site of each antibody. Based on the experimental data, the formation of the immunocomplexes will provide a general and convenient method for suppression of aggregation and stabilization of the target molecules without affecting the biological properties of the

given target molecule. The present invention uses genetically engineered antibodies obtained from such selected antibodies as protecting agents of in vivo aggregation of their antigen, leading to production of a soluble and stabilized protein..

Protein aggregation is of major importance that extends into mechanisms of human diseases and fundamental aspects of protein folding, expression and function. Data in literature (De Young et al., 1993; Wetzel, 1994; Wetzel, 1991) suggests that aggregation is non-specific in the sense that addition of other proteins can influence the extent of aggregation of a certain protein. However, the specificity can be related to a particular residue or group of residues which play a special role in the folding-related aggregation of a polypeptide (Silen and Agard, 1989; Zhu et al. 1989; Winter et al., 1994; Brems 1988). The identification of such classes of sequences that play a role in the folding-unfolding and/or solubilization-aggregation provides the basis of the present invention for the prevention of aggregation.

Stabilization procedures based on protein-protein recognition processes, fundamental to biology, have been previously investigated (Chothia and Janin, 1975; Jaenicke, 1991). Introduction of molecular chaperones which enable folding and stabilization of unrelated proteins appears to be tailored to prevent misfolding and aggregation at an early stage during folding. However, the central problem remaining in in vivo folding is how to efficiently prevent aggregation without blocking the forward pathway of correct folding and biological activity of the native state (Ellis et al. 1991; Gething and Sambrook, 1992; Hendrick and Hartl, 1993).

The availability of monoclonal antibodies (mAbs) led to a better understanding of the effect of highly specific antigen-antibody interactions on the antigen or target molecule behavior. The complementary conformation between the interacting regions of the antibody with its antigen confers the high specificity and stability to the immunocomplex formed (Goldberg, 1991). Properly selected monoclonal antibodies, unlike the ubiquitous nature of the chaperones, bind to a specific antigen at a distinct and preselected antigenic site without interfering in the biological activity of the antigen and assist in antigen refolding (Blond and Goldberg, 1987; Carslon and Yarnush, 1992; Solomon and Schwartz, 1995).

The present invention utilized the effect of immunocomplexation in the suppression of antigen aggregation using as a model system the interaction of Carboxypeptidase A (CPA) and its monoclonal antibodies. CPA occupies a prominent position in the literature of metalloenzymes, being a well-characterized zinc exopeptidase that exhibits both peptidase and esterase activity (Vallee and Galdes, 1984). A large number of mAbs were prepared by the application towards native enzymes (Solomon et al. 1984) and their properties were widely investigated. Some of these antibodies bind to the enzyme with a relatively high binding constant, remote from its active site and assist in refolding of already heat denatured enzyme (Solomon and Schwartz, 1995). ELISA measurements and determination of residual enzymic activity as a probe of native structure are used to monitor the effect of two different mAbs, namely CP_{10} and CP_9 on the inhibition of CPA aggregation.

The above discussion provides a factual basis for the use of monoclonal antibodies and genetically engineered antibody fragments as therapeutics for the prevention of protein aggregation. The methods used with and the utility of the present invention can be shown by the following examples.

EXAMPLES

METHODS AND REAGENTS

Carboxypeptidase A (CPA)

CPA was obtained as an aqueous crystalline suspension (Sigma Chemical Co., St. Louis, Mo.). The crystals were washed with double-distilled water, centrifuged, and dissolved in 0.05M Tris-HCl/0.5M NaCl buffer, pH 7.5. Insoluble material was removed by centrifugation. The enzyme concentration was derived from the absorbance at 278 nm.

Determination of CPA Enzymatic Activity

The enzymatic activities of CPA and its immunocomplexes were determined spectrophotometrically at 254 nm using either 1 mM hippuryl-L-phenylalanine as peptidase substrate or hippuryl-DL-β-phenyllactic acid as esterase substrate in 0.5M NaCl/0.05M Tris-Hl, pH 7.5, (Solomon et al., 1989).

Amyloid

Amyloid peptides, Aβ 1–40 (Cat. No. A-5813) and Aβ 1–28 (Cat. No. A-1084) corresponding to amino acids 1–40 and 1–28 of Aβ respectively, were purchased from Sigma Chemical Co., St. Louis, Mo., USA).

Amyloid solutions were prepared by dissolving the peptides in water at concentration of 10 mg/ml. The stock solution was stored in aliquotes at –20° C.

Aggregating agents

Heparan sulfate (Cat. No. H 5393) was purchased from Sigma Chemical Co., St. Louis, Mo., USA). Stock solutions of metal chlorides were made up from dry salts at concentration of 1 mM in TRIS pH 7.4.

Monoclonal Antibody Production

In general, monoclonal antibodies may be prepared against a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof may be isolated and used as the immunogen. Such proteins or peptides can be used to produce monoclonals by standard production technology well known to those skilled in the art as further described generally in Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988 and Milstein (1980). Briefly, mouse monoclonal antibodies were prepared by hyperimmunization of an appropriate donor with the protein or peptide fragment, generally a mouse, and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

The harvested monoclonal antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, Oxford, 1982. The binding of antibodies to a solid support substrate is also well known in the art. (see for a general discussion Harlow & Lane Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Publications, New York, 1988) The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, β-galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.

Alternatively, commercially available antibodies can be used. α-Human β-amyloid 6F/3D was obtained from ACCURATE Chemical and Scientific Corp. (Westbury, N.Y., USA). mAb AMY 33 was purchased from ZYMED San Francisco, Calif., USA. A polyclonal, affinity purified rabbit IgG obtained against the synthetic Alzheimer β-amyloid (Cat. No. 1381431) was purchased from Boehringer-Mannheim, GmbH, Germany.

Purification and characterization of anti-CPA mAbs

The monoclonal antibodies, CP-10, CP-9, which interact with CPA at high binding constants, were selected for further study. The preparation and characterization of the monoclonal antibodies CP_{10} and CP_9 (chosen for the present study) were previously described (Solomon et al., 1989; Solomon and Balas, 1991).

These antibodies were isolated and purified by affinity chromatography on protein A-Sepharose from the corresponding ascites fluids according to Harlow and Lane.

Protocol for Determining Effect of Monoclonal Antibody Binding on CPA Activity

CPA (1 mg/ml) was incubated at 50° C. in the absence and in the presence of increasing amounts of mAbs CP_{10} and CP_9 (100 μl in PBS) ranged between 0–2 molar ratio antibody/CPA. The enzymic activities of the immunocomplexes formed were measured as described herein above. Data related in percentage, 100% being considered the enzymic activity of CPA before denaturation.

ELISA Tests

The antigen-coating solutions (100 μl containing native CPA (10–25 μl ml) in PBS, pH 7.4, were incubated overnight at 4° C. in a polystyrene ELISA plate (Costar, Cambridge, Mass.). Diluted ascites fluid (0.1 ml) containing the desired mAb (1:2000 to 1:18,000 v/v in PBS) was added and incubated at 37° C. for 1 hour. The amount of bound mAb was determined with β-galactosidase-linked $F(ab)_2$ fragments of sheep anti-mouse IgG (Amersham International, UK).

The quantitation of the amount of aggregated CPA during denaturation at 50° C. was determined by competitive and sandwich ELISA, as follows:

Competitive ELISA Assays

CPA (10 μl/ml of PBS) was adsorbed onto ELISA plates overnight at 4° C., the remaining active groups on the plate being blocked with non-fat milk. To the soluble CPA (200 ng in 10 μl PBS), incubated for one hour at 50° C., the mAb CP_{10} (molar ratio 1:1 Ab/CPA) was added and allowed to interact with the remaining soluble CPA for one hour at 37° C. In parallel, the mAb was added to the CPA solutions before exposure at 50° C. for one hour. After incubation, the CPA preparations were removed by centrifugation at 15,000 rmp for 15 minutes and applied on the ELISA plates coated with CPA. The antibody which did not bind to soluble CPA in the reaction mixture will bind to the coated CPA; the amount of antibody bound to the coated antigen will be conversely proportional to the extent of CPA aggregation and determined using α-mouse antibodies labeled with horseradish peroxidase (HRP). The color developed by HRP (0-phenylenediamine (OPD) as substrate) was measured at OD_{495} using an ELISA plate reader. The amount of antibody bound on the coated CPA in the absence of soluble CPA was considered as 100%.

Sandwich ELISA

The ELISA plates were coated with rabbit polyclonal antibodies raised against CPA (1 μl/well) by incubation at 37° C. for two hours. The residual active groups were blocked by non-fat milk. Soluble CPA (200 ng in 10 μl PBS) was exposed to 50° C. for one hour and the aggregated CPA

was removed by centrifugation at 15,000 g for 15 minutes. The residual soluble CPA was incubated for another one hour at 37° C. with mAb CP_{10} and mAb CP_9 at various molar ratio antibody/antigen. In another set of experiments, the mAbs were added to the reaction mixtures before incubation at 50° C. and then exposed for one hour at 50° C. After the incubation period, all the immunocomplexed CPA preparations were centrifuged and added to the ELISA plate, previously coated with polyclonal CPA antibodies, for 12 hours at 4° C. The amount of mAb bound, determined as described above, will be proportional to the amount of soluble CPA which remained after exposure to aggregation conditions. The results are presented in percentages, 100% being the maximal absorbance obtained before CPA heat treatment.

All data presented are the mean of triplicate determinations. The standard deviation of the intra-assay and interassays were less than 5% in all cases.

Amyloid ELISA Assays

The ELISA plates were coated with rabbit polyclonal antibodies (Boeringer-Mannheim) raised against synthetic α-amyloid (1–40) (Sigma) (100 ng/well) via covalent attachment to epoxy-coated ELISA plates by incubation at 4° C. for 16 hours. The residual epoxy groups were blocked by non-fat milk. The reaction mixtures containing aqueous solution of α-amyloid (100 ng/ml), heparan sulfate (50 mM) and/or chloride metal solutions (10^{-3}M at pH 6.5), were incubated at 37° C. for three hours. The aggregated β-amyloid preparations were removed by centrifugation at 15,000 g for 15 minutes. The residual soluble β-amyloid was incubated for another one hour at 37° C. with mAbs AMY 33 and/or 6F3D at equal molar ratio antibody/antigen. In another set of experiments, the mAbs were added to the reaction mixtures before incubation at 37° C. and then incubated together for 3 hours at 37° C. After the incubation period, the immunocomplexed amyloid preparations were added to the ELISA plates, previously coated with polyclonal anti-amyloid antibodies. The amount of mAb bound will be proportional to the amount of soluble amyloid which remained after exposure to aggregation conditions.

The amount of bound antibody was determined using a-mouse second antibodies labeled with horseradish peroxidase (HRP). The enzyme activity of HRP is directly proportional with the amount of residual amyloid bound to rabbit polyclonal antibodies. The enzyme activity of HRP was measured using O-phenylenediamine (OPD) as substrate. The color developed was measured at A_{495} using an ELISA reader. Data represent the mean of triplicate determinations. The standard deviation of the intra-assay and interassays were less than 5% in all cases.

EXAMPLE 1

Aggregation of heat denatured CPA was followed by determination of the residual enzymic activity of CPA using esterase and peptidase substrates. CPA (1 mg/ml) was incubated at various temperatures for one hour, and residual enzymic activity was determined. The temperature of 50° C. was chosen for further study. At this temperature, mAbs studied keep all their immunological activity (personal data). Effect of immunocomplexation of CPA with its mAbs was monitored by: (1) Determination of enzymic activity and (2) ELISA measurements as described herein above.

Monoclonal antibodies raised against native antigens proved to be powerful tools in identification and characterization of folding steps by recognition of incompletely folded antigens (Mendrick and Hartl, 1993). The selected antibodies might interact at sites where protein unfolding is

initiated, thereby stabilizing the protein and suppressing further aggregation.

The main difference between mAbs and molecular chaperones is that the latter does not bind to native proteins and is capable of interacting with many different polypeptide chains without exhibiting an apparent sequence preference (Goloubinof et al., 1989). Moreover, chaperones suppress aggregation but do not redissolve aggregate already present.

The aggregation of CPA and loss of its enzymic activity was found to be dependent on the temperature and the time of incubation (FIGS. 1, 2). Esterase activity seems to be more affected at higher temperature than peptidase activity, indicating that these activities follow different reaction mechanisms (FIG. 2). These data are compatible with applicant's previous results (Solomon et al., 1989; Solomon and Balas, 1991), as well as with the findings of Vallee and his collaborators (1969), who postulate that the active site of CPA consists of non-identical but interacting binding sites for peptides and ester substrates. As shown in FIG. 2, the immunological recognition of partially heat denatured enzyme is better conserved than its residual enzymic activity.

The inhibition of CPA aggregation, induced by incubation at 50° C. for one hour by its interaction with two mAbs, CP_9 and CP_{10}, was followed by measuring the peptidase and esterase enzymic activities (FIG. 3). The two mAbs, CP_{10} and CP_9 were chosen for this study on the basis of previous data regarding their effect on the enzyme behavior (Solomon and Schwartz, 1995; Solomon et al., 1989; Solomon and Balas, 1991). The protection of enzymic activity of heated CPA was dependent on the amount of antibody added to the enzyme and a molar ratio of 1:1 antibody/enzyme was sufficient for the maximum protection effect. The peptidase activity of the CPA-CP_{10} complex was maintained at 90% of its initial activity in the presence of mAb CP_{10}. The protective effect of mAbs on CPA activity during heat denaturation was found to be related to the location of the antigenic site of each antibody (FIG. 4). Even a great excess of unrelated antibody did not assist in maintaining CPA activity. Increase in preservation of enzyme activity can be reached, however, in the presence of a pair of two antibodies. This effect seems to be the result of a "locking" of the conformation caused by simultaneous interaction with two different antibodies at two distinct epitopes (Solomon and Balas, 1991).

The amount of aggregated CPA was quantitated by ELISA measurements. Disappearance of CPA, as a result of its aggregation during incubation for one hour at 50° C., was followed by a competitive ELISA assay (FIG. 5) and a sandwich assay (FIG. 6). The mAb, CP_{10}, maintained 100% of the CPA activity in solution during heating for one hour at 50° C. (FIG. 6); CP_9 provided a slight effect on CPA protection at 50° C. Both antibodies prevent the aggregation of CPA, similar to the data shown in FIG. 4, recognizing "key positions" on the molecule responsible for heat denaturation and aggregation of CPA.

The biological activity of the enzyme seems to be more sensitive to high temperatures than the insolubilization process. Subtle heat-induced conformational changes occurring in CPA molecules are reflected by change in enzymic activity, even before transition between native-molten globule conformation-aggregated states occurred. These findings are in contradiction to previous suggestions that the biological function of a protein does not necessarily require fully folded protein (Hattori et al., 1993).

The antigen binding site of mAb CP_{10} (previously named CP_{10}) was identified as one of the immunodominant regions

of the enzyme, localized on the surface of the molecule between amino acids 209–218 (Solomon et al. 1989). The localization of the epitope recognized by CP_9 has not yet been clarified, but it does not interfere with the mAb CP_{10} during simultaneous binding to CPA molecule, as suggested by additivity measurements (Solomon and Balas, 1991).

Similar effects in suppression of antigen aggregation were obtained after immunocomplexation of horseradish peroxidase.

The data available in literature suggests that for practically all the antigens it might be possible to prepare monoclonal antibodies which bind with high affinity without affecting their catalytic activity. Moreover, mAbs like the majority of immunoglobulins, are robust molecules and survive in a variety of environments, including high temperatures, low pH, denaturing agents. Formation of such immunocomplexes should provide a general and convenient method for suppression of aggregation and stabilization of their antigen without affecting the biological properties of the given antigen.

EXAMPLE 2

This example investigates the immunocomplexation effect on the in vitro aggregation of β-amyloid. Aggregation of β-amyloid was found to be dependent on the pH, peptide concentration, temperature and time of incubation (Burdick et al., 1992). In applicant's experiments, the aggregation of β-amyloid was performed by incubation of aqueous solution of βA4 (10 mg/ml) for three hours at 37° C. The β-amyloid aggregation was followed by ELISA measurements using two different commercially available monoclonal antibodies raised against β-amyloid: α-human β-amyloid 6F/3D obtained from Accurate Chemical and Scientific Corp, Westbury, N.J. USA, and mAb AMY 33 (Stern et al., 1990), purchased from Zymed, San Francisco, Calif., USA, raised against peptides 8–17 and 1–28, respectively, of the β-amyloid.

The addition of the antibodies was made before or after exposure of synthetic β-amyloid to the aggregation process (FIG. 7A, B). The aggregation of the β-amyloid was performed in the presence of heparan sulfate and/or metal ions, such as Zn^{2+} and Al^{3+}. The antibody AMY-33, which is supposed to recognize an epitope spanned between the sequence 1–28, inhibits the β-amyloid aggregation occurring in the presence or absence of heparan sulfate (FIG. 7A). Any significant effect on metal-induced amyloid aggregation was observed under the same experimental conditions. The mAb 6F/3D, recognizing an epitope located between the sequence 8–17 of the β-amyloid, interferes with Zn^{2+}-induced aggregation, showing a partial solubilization effect on already aggregated β-amyloid, but has no effect on other aggregating agents (FIG. 7B).

Metals, such as Zn^{2+} and Al^{3+}, have been proposed as risk factors for Alzheimer's disease development (Mantyh et al., 1993; Frederickson, 1989; McLachlan et al., 1991). The aggregation of βA4 induced by aluminum is distinguishable from that induced by Zn in terms of role, extent, pH and temperature dependence (Mantyh et al. 1993). Although the precise site of interaction of metal ions and βA4 is not clarified, several residues in βA4 are candidates for metal binding. The βA4 histidine residues (His_{13}-His_{14}) may be implicated in fibril formation and it is conceivable that at least H_{14} remains available for intermolecular electrostatic interactions between anti-parallel chains (Talfous et al., 1994). The site defined by Val_{12}-His_{13}-His_{14}-Glu_{15}-Lys_{16}-Leu_{17} has been identified as a sequence containing a heparan

sulfate binding domain (Fraser et al., 1992) and His_{13} and Lys_{16} are supposed to provide the cationic binding sites being exposed on the same face of the peptide β sheet (Talafous et al. 1994).

Binding of mAb AMY-33 to βA4 prevents self-aggregation of the β-amyloid, probably by recognizing the sequence 25–28 located in the proposed aggregation fragment comprising the amino acids between 25–28 (Yankher et al., 1990) (FIG. 8). This antibody prevents intramolecular aggregation occurring in the presence of heparan sulfate, which is supposed to affect only the aggregation of preexisting amyloid fibers (Fraser et al., 1992). Inhibition of β-amyloid aggregation in the presence of mAb 6F/3D was partially effective only in the presence of Zn^{2+}.

On the basis of applicants findings regarding other antigen-antibody systems studies (Solomon et al., 1989; Solomon and Balas, 1991), the formation of the immunocomplexes with selected, highly specific monoclonal antibodies, should provide a general and convenient method to prevent aggregation of the proteins without affecting their biological properties.

At least 15 different polypeptides are known to be capable of causing in vivo different forms of amyloidosis via their deposition in particular organs or tissues as insoluble protein fibrils.

Recent advances in antibody engineering technology, as well as in the development of suitable delivery systems (Haber, 1992; Pluckthun, 1992; Travis, 1993; Marasco et al., 1993) make it possible to develop functional small antibody fragments to serve as therapeutic chaperones for the treatment of Alzheimer's disease as well as other human amyloidosis diseases by gene based therapies.

Application of the above findings for in vivo aggregation, can confer to single chain antibodies (Pluckthun, 1992) or other engineered antibody fragments, a protective role in the renaturation of recombinant proteins.

Throughout this application various publications are referenced by citation or number. Full citations for the publications referenced by number are listed below. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

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What is claimed is:

1. A method of selecting an anti-aggregation molecule having the chaperone-like activity of anti-aggregation, wherein the anti-aggregation molecule is selected from the group consisting of a monoclonal antibody, a genetically engineered antibody antigen binding fragment, and a single chain monoclonal antibody, and wherein said anti-aggregation molecule binds to a bioactive native target polypeptide epitope with a high binding constant and is non-inhibitory to the biological activity of the target polypeptide comprising the steps of:

denaturing a target polypeptide which aggregates,

mixing the target polypeptide with said anti-aggregation molecule to form a mixture,

incubating the mixture under conditions allowing for aggregation,

selecting non-aggregated mixtures, and

testing the nonaggregated target polypeptide coupled to the anti-aggregation molecule for bioactivity thereby selecting an anti-aggregation molecule with the chaperone-like activity of anti-aggregation which when coupled to the target polypeptide maintains bioactivity.

2. The method of claim 1 further characterized by the target polypeptide being β-amyloid.

3. A method of selecting an anti-aggregation molecule having the chaperone-like activity of anti-aggregation, wherein the anti-aggregation molecule is selected from the group consisting of a monoclonal antibody, a genetically engineered antibody antigen binding fragment, and a single chain monoclonal antibody, and wherein said anti-aggregation molecule binds to a bioactive native target polypeptide epitope with a high binding constant, reverses aggregation and is non-inhibitory to the biological activity of the target polypeptide comprising the steps of:

preparing an aggregated target polypeptide,

mixing the target polypeptide with said anti-aggregation molecule to form a mixture,

selecting mixtures with non-aggregated target polypeptides, and

testing the target polypeptide coupled to the anti-aggregation molecule for bioactivity thereby identifying an anti-aggregation molecule with the chaperone-like activity of anti-aggregation which when coupled to the target polypeptide maintains bioactivity.

4. The method of claim 3 further characterized by the target polypeptide being β-amyloid.

* * * * *

[57] **ABSTRACT**

A method of selecting anti-aggregation molecules with chaperone-like activity that have characteristics including binding to a native target molecule epitope with a high binding constant and are non-inhibitory to the biological activity of the target molecule. The method [molecules] involves denaturating a target molecule in the presence of presumptative antiaggregation molecules to prevent the target molecules from self-or induced-aggregation. The nonaggregated target molecule coupled to the anti-aggregation molecule is then tested for bioactivity.